Analytica Chimica Acta 637 (2009) 196–207

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

New method for the analysis of flukicide and other anthelmintic residues in bovine milk and liver using liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form 28 October 2008
Accepted 29 October 2008
Available online 6 November 2008

Keywords:
Anthelmintics
Flukicides
Residues
Food
Liquid chromatography–tandem mass spectrometry
QuEChERS

ABSTRACT

A liquid chromatographic-tandem mass spectrometric (LC–MS/MS) multi-residue method for the simultaneous quantification and identification of 38 residues of the most widely used anthelmintic veterinary drugs (including benzimidazoles, macrocyclic lactones, and flukicides) in milk and liver has been developed and validated. For sample preparation, we used a simple modification of the QuEChERS method, which was initially developed for pesticide residue analysis. The method involved extracting sample (10 g) with acetonitrile (10 mL), followed by phase separation from water (salting out) with $MgSO_4$:NaCl (4:1, w/w). After centrifugation, an aliquot of the extract (1 mL) was purified by dispersive solid-phase extraction with $MgSO_4$ (150 mg) and C_{18} (50 mg), prior to LC–MS/MS analysis. Two injections of the same extract were required with the LC–MS/MS instrument to cover the 30 electrospray positive and 8 electrospray negative analytes. The limit of quantitation of the method was 5 $\mu g\,kg^{-1}$ for 37 analytes (and 10 $\mu g\,kg^{-1}$ for dichlorvos). The method was successfully validated according to the 2002/657/EC guidelines. Recovery of analytes was typically in the 70–120% range, with repeatabilities and reproducibilities typically <15% in milk and <20% in liver.

1. Introduction

Helminths are the single most important group of parasitic infections in livestock [1]; and include three types—cestodes (tapeworms), nematodes (roundworms) and trematodes (liver flukes). Helminth infections are controlled through the administration of anthelmintic agents, which are essential for maintaining high weight gains and reproductive performance in livestock [2]. During the 1980s, there was a shift from therapeutic treatment (salvage) to chemoprophylactic control of infections [3]. Chemoprophylaxis was supported by widespread availability of anthelmintics, due to the availability of over-the-counter veterinary medicines and the launch of cheaper generic products. This has led to concerns that anthelmintics are often unnecessarily administered to animals, leading to an increased risk of residues occurring in food and due to the build-up of resistance to the dwindling numbers of useful drugs.

Maximum residue limits (MRLs) have been set for a number of anthelmintics in milk and edible tissues (muscle, liver, kidney and fat) with the aim of minimizing the risk to human health associated with their consumption (Table 1). In general, it can be concluded that anthelmintic residues pose no human health risk if veterinary drugs are properly administered and the recommended doses are correctly adhered to. However, there may be a concern that if withdrawal periods are not adhered to, or if products are administered to animals in unapproved applications (e.g. administration to lactating species) that levels may exceed MRLs in foods. Some of these drugs possess toxicological properties namely teratogenicity and embryotoxicity [4]; neurotoxicity [5], hyperplasia, [6], goitrogenicity [7] and mutagenicity [8].

The major anthelmintics (including wormers, flukicides and certain pesticides) and the structures of their key residues are shown in Fig. 1. The analysis of a number of these substances, namely benzimidazoles (BZs) and macrocyclic lactones (MLs) was recently reviewed in detail [9,10]. These review papers highlighted shortcomings in published methodologies for analysing BZ residues in food, in particular the failure to include several key residues in multi-residue methods. In addition, there is a general lack of

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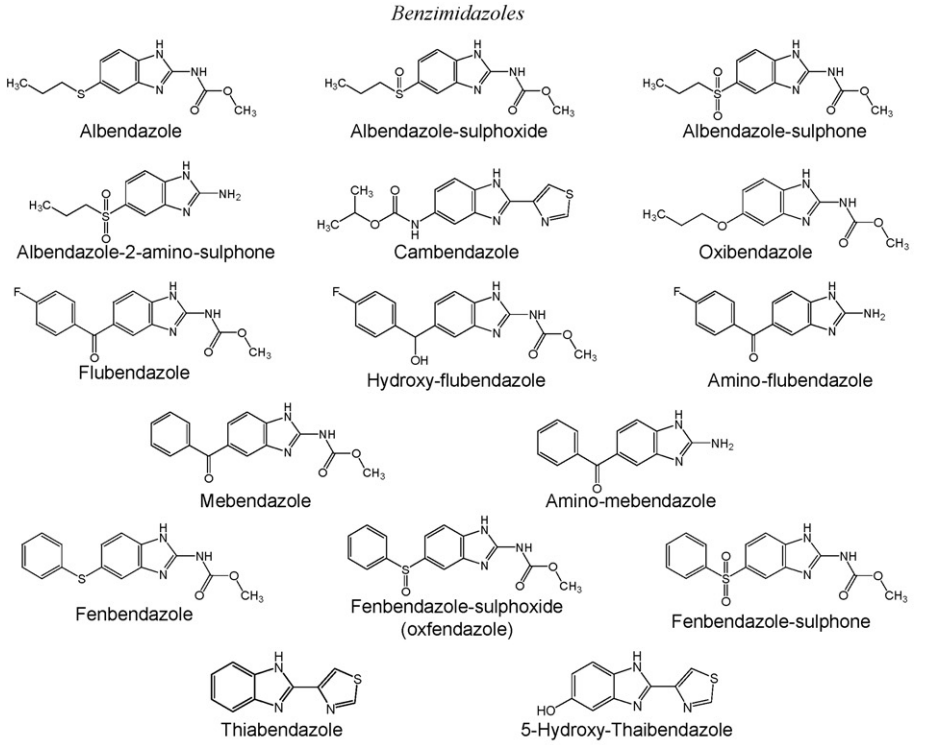
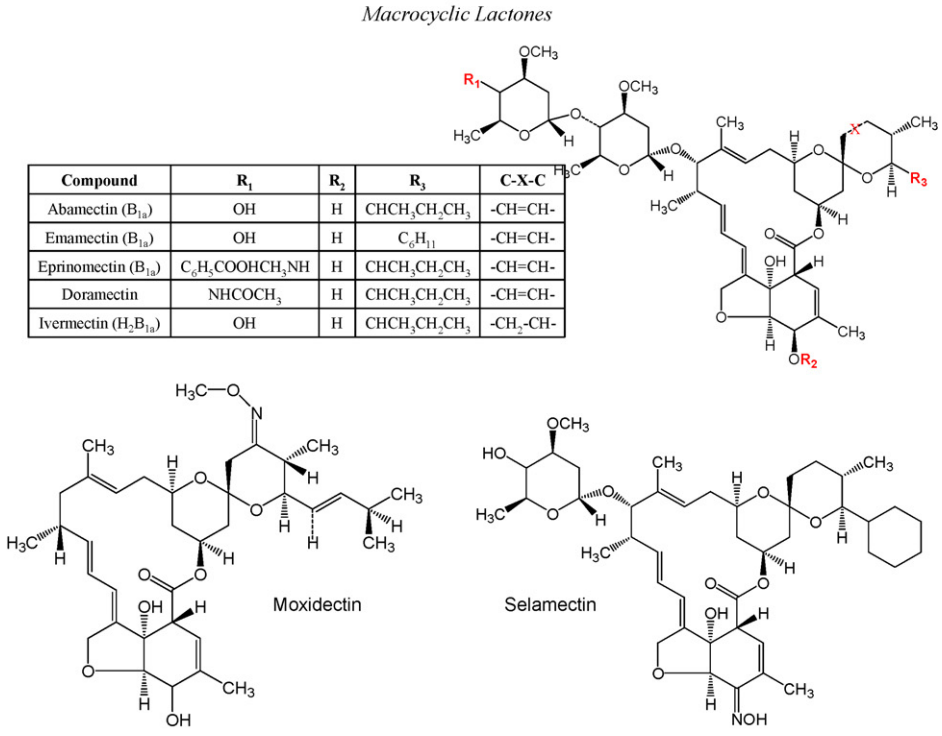
doi:10.1016/j.aca.2008.10.072

Table 1
The permitted limits of the 38 anthelmintic residues, and 1× fortified concentration used in the study.

Analyte	Type	MRL (EU)		Tolerance (US)		Codex		Fortified concentration	
		Milk ($\mu g\,kg^{-1}$)	Liver ($\mu g\,kg^{-1}$)	Milk ($\mu g\,kg^{-1}$)	Liver ($\mu g\,kg^{-1}$)	Milk ($\mu g\,kg^{-1}$)	Liver ($\mu g\,kg^{-1}$)	Milk ($\mu g\,kg^{-1}$)	Liver ($\mu g\,kg^{-1}$)
Bithionol	Flukicide							10	10
Clorsulon	Flukicide		100					10	100
Closantel	Flukicide		1000				1000	10	1000
Nitroxynil	Flukicide		20					10	20
Niclosamide	Flukicide							10	10
Rafoxanide	Flukicide		10					10	10
Oxyclozanide	Flukicide	10	500					10	500
Macrocyclic lactones									
Abamectin	Anthelmintic		20				100	10	20
Emamectin	Anthelmintic							10	10
Eprinomectin	Anthelmintic	20	1500	12	480	20	2000	20	1500
Doramectin	Anthelmintic		100		100	10	100	10	100
Ivermectin	Anthelmintic		100		100	10	100	10	100
Moxidectin	Anthelmintic	40	100	40	200		100	40	100
Selamectin	Anthelmintic							10	10
Benzimidazoles									
Albendazole	Anthelmintic	100	1000					100	1000
Albendazole-sulfoxide	Anthelmintic	100	1000					100	1000
Albendazole-sulfone	Anthelmintic	100	1000					100	1000
Albendazole-2-amino-sulfone	Anthelmintic	100	1000		200			100	1000
Cambendazole	Anthelmintic							10	10
Fenbendazole	Anthelmintic	10	500			100	500	10	500
Fenbendazole-sulfoxide	Anthelmintic	10	500	600	800	100	500	10	500
Fenbendazole-sulfone	Anthelmintic	10	500			100	500	10	500
Flubendazole	Anthelmintic							10	10
Amino-flubendazole	Anthelmintic							10	10
Hydroxy-flubendazole	Anthelmintic							10	10
Mebendazole	Anthelmintic							10	10
Amino-mebendazole	Anthelmintic							10	10
Oxibendazole	Anthelmintic							10	10
Thiabendazole	Anthelmintic	100	100	50	100	100	100	100	100
5-Hydroxy-thiabendazole	Anthelmintic	100	100					100	100
Triclabendazole	Flukicide		250			300		10	250
Triclabendazole-sulfoxide	Flukicide		250					10	250
Other									
Coumaphos	Pesticide							10	10
Coumaphos-oxon	Pesticide							10	10
Dichlorvos	Pesticide							10	10
Haloxon	Anthelmintic				100			10	10
Levamisole	Anthelmintic		100		100		100	10	100
Morantel	Anthelmintic	50	800		700			50	800

suitable multi-residue methods for detecting the non-BZ or -ML anthelmintics listed in Table 1. Only two multi-residue methods have been reported in the literature for the detection of flukicide residues. Takeba et al. reported the determination of five flukicide residues in milk by liquid chromatography (LC) with electrochemical detection [11]. The method included bithionol, oxyclozanide, dephosphate-bromofenofos, DS-6 and tribromsalan; the latter three drugs are not in fact registered for use in the EU. The limits of detection of this method were between 4 and 20 $\mu g\,kg^{-1}$. Von Tonder et al. reported an LC-UV method for the analysis of niclosamide, oxyclozanide, albendazole and fenbendazole drugs in veterinary formulations [12]. Several methods have been reported





Compound	R_1	R_2	R_3	C-X-C
Abamectin (B_{1a})	OH	H	$CHCH_3CH_2CH_3$	-CH=CH-
Emamectin (B_{1a})	OH	H	C_6H_{11}	-CH=CH-
Eprinomectin (B_{1a})	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Doramectin	$NHCOCH_3$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Ivermectin (H_2B_{1a})	OH	H	$CHCH_3CH_2CH_3$	$-CH_2$-CH-

Fig. 1. Structures of all 38 anthelmintics in the study.

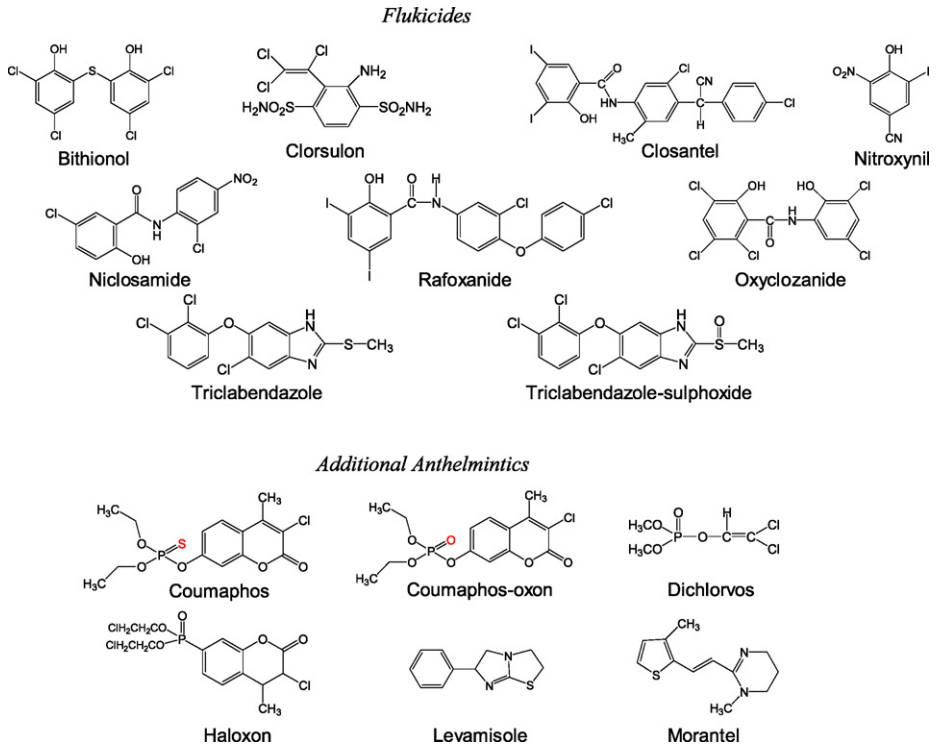


Fig. 1. (*Continued*)

for the determination of individual flukicides and/or in combination with a single BZ or ML residue [13–19].

All of the methods mentioned above involve lengthy, labour-intensive, and expensive extraction methods that generate copious amounts of solvent waste. In addition to meeting acceptable performance criteria for the application, a multi-residue method should be fast, inexpensive, easy to perform and require a minimal quantity of chemicals, space and labware, as well as delivering a high degree of robustness and cover a wide range of analytes and matrices. Most sample preparation methods involve many multi-step procedures, but if the number of steps can be streamlined using a simpler procedure, precision and accuracy are typically improved along with time, labour and cost savings.

To achieve these objectives Anastassiades et al. developed a new method for the sample preparation of pesticide residues in fruits and vegetables [20]. The resulting sample preparation method was termed QuEChERS (standing for quick, easy, cheap, effective, rugged and safe), and since its inception, it has been successfully used to analyze hundreds of pesticides in a variety of foods [21].

In QuEChERS, the high-moisture sample (water is added to dry foods) is first extracted with an organic solvent (acetonitrile (MeCN), ethyl acetate, or acetone) in the presence of salts ($MgSO_4$, NaCl, and or buffering agents) to induce phase separation of the solvent from the water. Upon shaking and centrifugation, an aliquot of the organic phase is subjected to further cleanup using dispersive-SPE, which entails mixing sorbents with the extract. Afterwards, the mixture is centrifuged and the resulting supernatant can be analyzed directly or can be subjected to a concentration and/or solvent exchange step if necessary. The approach is very flexible, and since its development [20] there have been several modifications to the technique depending on analytes, matrices, instrumentation, and analyst preferences [21–28]. The approach uses very little labware and generates little waste. The technique provides high recovery for many LC- and GC-amenable analytes, gives high reproducibility, and costs less than many typical sample preparation approaches [20].

In the past, LC-UV or LC-fluorescence was the primary analytical tool used to detect anthelmintics. However, these detectors typically require extensive cleanup of the extracts, detect a limited number of analytes and are prone to interferences. Furthermore, fluorescence detection often requires derivatization of analytes. In recent times, residue chemists are increasingly switching to LC–MS/MS to qualitatively and quantitatively detect veterinary drug residues [29]. LC–MS/MS commonly enables the determination of drugs with high molecular masses as well as polar, non-volatile and thermally labile analytes without derivatization. However, for the analysis of complex matrices such as liver, kidney or muscle commonly used in veterinary drug residue analysis, an appropriate sample preparation step is necessary to minimize matrix effects in the ionisation process. Yet, due to the high selectivity of the tandem mass spectrometer, the sample cleanup can be kept relatively simple. The ongoing development of LC–MS/MS has opened new strategies for veterinary drug screening, particularly in the area of multi-class multi-residue methods. Triple quadrupoles are the most widely used instruments for modern residue analysis and provide excellent performance, allow for the simultaneous analysis of >100 analytes in a short time period [30].

The objective of this study was to develop and validate a simple but effective method for the detection and quantification of 38 anthelmintic residues in cattle milk and liver. The method was validated according to the 2002/657/EC [31] guidelines as required for EU monitoring programs. The factors investigated included recovery, selectivity, precision, linearity and analytical limits, including the decision limits (CCα) and detection capabilities (CCβ) of the method. This method is the first to report the detection of 38 residues of the most widely used anthelmintic drugs (including nine flukicides) in biological samples.

2. Experimental

2.1. Reagents and chemicals and samples

Acetonitrile and methanol (MeOH) solvents (HPLC grade), octadecylsilane (C_{18}, Bakerbond, 40 μm Prep LC packing), and NaCl and ammonium acetate (analytical grade) were obtained from Mallinckrodt-Baker (Phillipsburg, NJ, USA). MS-grade formic acid and ammonium formate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Primary secondary amine (PSA) sorbent (40 μm, analytical grade) was obtained from Varian (Palo Alto, CA, USA), and graphitized carbon black (GCB) was from Supelco (Bellefont, PA, USA). Pre-weighed mixtures of 50 mg combinations each of PSA, GCB, and/or C_{18} sorbents plus 150 mg anhydrous magnesium sulphate ($MgSO_4$) in 2 mL mini-centrifuge tubes, and 5 g portions of $MgSO_4$:NaCl (4:1, w/w) in 50 mL centrifuge tubes, and $MgSO_4$ were obtained from UCT (Bristol, PA, USA). Water was purified by a Barnstead E-pure water purification system (Dubuque, IA, USA). Liver samples, free of residues, were obtained from a local organic farm, while organic milk was readily available from most supermarkets. All samples were tested for residues prior to method development.

2.2. Standards and stock solutions

Abamectin, albendazole, bithionol, clorsulon, closantel, coumaphos, dichlorvos, doramectin, emamectin, fenbendazole, haloxon, ivermectin, levamisole, mebendazole, nitroxynil, niclosamide, morantel, oxyclozanide, rafoxanide and thiabendazole were obtained from Sigma–Aldrich (St. Louis, MO, USA). Albendazole-sulphoxide, albendazole-sulphone, albendazole-2-amino-sulphone, 5-hydroxy-thiabendazole, triclabendazole and triclabendazole-sulphoxide were purchased from Witega Laboratorien Berlin-Aldershof GmbH (Berlin, Germany). Coumaphos-oxon was obtained from the Environmental Protection Agency's National Pesticide Repository (Fort Meade, MD, USA). Fenbendazole-sulphoxide (oxfendazole), oxibendazole and cambendazole were purchased from QMX Laboratories (Thaxted, UK). Eprinomectin was donated by Merial (Lyon, France) and fenbendazole-sulphone was donated by Hoechst AG (Frankfurt, Germany). Flubendazole, amino-flubendazole, hydroxy-flubendazole and amino-mebendazole were donated by Janssen Animal Health (Beerse, Belgium); selamectin was donated by Pfizer Animal Health (New York, NY, USA) and moxidectin was donated by Fort Dodge Animal Health (Southampton, UK).

Primary stock solutions were prepared at a concentration of 1000 μg mL^{-1} in either MeOH or MeCN, while albendazole and cambendazole were prepared at a concentration of 500 μg mL^{-1}; albendazole-sulphoxide, albendazole-sulphone, fenbendazole, fenbendazole-sulphone and hydroxy-flubendazole were prepared at a concentration of 200 μg mL^{-1}; mebendazole and flubendazole were prepared at a concentration of 100 μg mL^{-1}. All standard solutions were stored at −18 °C.

A 10 μg mL^{-1} working standard solution with all 39 analytes was prepared by direct dilution of the primary stock standard solutions in 10 mL MeCN. A 1 in 10 dilution of this standard was carried out to obtain a 1 μg mL^{-1} working standard solution.

Standard solutions for validation studies were prepared by direct dilution of primary stock standard solutions in 25 mL of MeCN. The liver validation solution was prepared at concentrations of 0.4 μg mL^{-1} (rafoxanide, bithionol, niclosamide, emamectin, selamectin, cambendazole, flubendazole, hydroxyl-flubendazole, amino-flubendazole, mebendazole, amino-mebendazole, oxibendazole, dichlorvos, coumaphos, coumaphos-oxon and haloxon); 0.8 μg mL^{-1} (nitroxynil); 4 μg mL^{-1} (clorsulon, doramectin, ivermectin, moxidectin, thiabendazole, 5-hydroxy-thiabendazole and levamisole); 10 μg mL^{-1} (triclabendazole and triclabendazole-sulphoxide); 20 μg mL^{-1} (oxyclozanide, fenbendazole, fenbendazole-sulphoxide and fenbendazole-sulphone); 32 μg mL^{-1} (morantel); 40 μg mL^{-1} (closantel, albendazole, albendazole-sulphoxide and albendazole-sulphone) and 60 μg mL^{-1} (eprinomectin).

The milk validation solution was prepared at concentrations of 1 μg mL^{-1} (oxyclozanide, fenbendazole, fenbendazole-sulphoxide, fenbendazole-sulphone and unapproved substances); 2 μg mL^{-1} (eprinomectin); 4 μg mL^{-1} (moxidectin); 5 μg mL^{-1} (morantel) and 10 μg mL^{-1} (albendazole, albendazole-sulphoxide, albendazole-sulphone, thiabendazole and 5-hydroxy-thiabendazole).

Triphenyl phosphate (TPP) and 2,4-dichlorophenoxyacetic acid (2,4-D) were purchased from Sigma–Aldrich, and were used as internal standards for positive and negative ion modes, respectively. A 10 μg mL^{-1} working internal standard solution was prepared by diluting 100 μL of the 1000 μg mL^{-1} standards in 10 mL of MeCN.

Cyprodinil (from Sigma–Aldrich) was used as a quality control standard to monitor the performance of the mass spectrometer. A 10 μg mL^{-1} working internal standard solution was prepared diluting 100 μL of 1000 μg mL^{-1} standard in 10 mL MeCN.

To obtain samples fortified at the four MRL levels: 50, 100, 150 and 200 μL of the milk validation standard was added to 10 g milk and 125, 250, 375 and 500 μL of the liver validation standard was added to 10 g liver.

To prepare matrix-matched standards for the calibration curves, three blank samples were extracted, cleaned-up and transferred (1 mL) into autosampler vials. To the vials were added either 5 μL of the 1 μg mL^{-1} mixed standard, 5 μL of the 10 μg mL^{-1} mixed standard or 50 μL of the 10 μg mL^{-1} mixed standard, which gave concentrations of 5, 50, and 500 μg kg^{-1}, respectively. 10 μL of internal standard (100 μg kg^{-1}) and QC standard (100 μg kg^{-1}) were also added to each vial.

2.3. Apparatus

A Sorvall Legend RT centrifuge (Thermo Fisher; Waltham, MA, USA), a Hill Scientific mv13 mini-centrifuge, and a Daigger Genie 2 vortex mixer (Vernon Hills, IL, USA) were used during sample preparation. The LC–MS/MS system consisted of an Agilent 1100 series LC (Santa Clara, CA, USA) including a binary pump, a vacuum degasser, column oven, and an autosampler, coupled with an API 3000 tandem quadrupole MS instrument (Applied Biosystems, Foster City, CA, USA) with an electrospray ion interface and divert valve placed between the column and source. The mass spectrometer was fully controlled by Analyst software Version 1.4.1. A syringe pump (Harvard Apparatus model 33, Holliston, MA, USA) connected to the interface was used for tuning purposes.

2.4. Sample preparation

Homogenised liver or milk samples (10 g) were weighed into 50 mL fluorinated ethylene propylene (FEP) centrifuge tubes. Samples were fortified with the analytes and/or the internal standards and let to stand for 15 min. Extraction was performed by adding MeCN (10 mL) followed by 5 g of $MgSO_4$:NaCl (4:1, w/w). Samples were immediately shaken for 1 min (to prevent agglomerates forming during $MgSO_4$ hydration) and centrifuged (3700 × *g*, 5 min). For sample cleanup, 1 mL of supernatant was transferred into a 2 mL mini-centrifuge tube containing $MgSO_4$ (150 mg) and C_{18} (50 mg). The sample extract was mixed by vortexing (1 min) and centrifuged (3000 × *g*, 2 min). An aliquot of the supernatant (500 μL) was transferred into an autosampler vial containing cyprodinil solution (QC standard, 5 μL)

Table 2
Summary of the retention times, diagnostic ions, and the MS/MS operating conditions for the 38 analytes and 3 internal standards.

Analyte	t_R (min)	Precursor ion (m/z)	Fragment ions (m/z)		Declustering potential (V)	Collision energy (eV) (most abundant ion)
			1[a]	2		
ESI$^+$						
Emamectin	11.0	886.5	158.1	126.0	26	49
Eprinomectin	12.4	914.5	186.0	154.1	16	29
Abamectin	12.9	890.5	305.1	567.2	21	35
Moxidectin	13.3	640.4	528.2	498.2	16	13
Doramectin	13.5	916.5	331.2	593.2	26	37
Ivermectin	14.8	892.5	569.2	307.1	11	21
Selamectin	14.9	770.4	333.2	113.1	46	31
Albendazole-2-amino-sulfone	7.8	240.0	133.0	197.8	31	39
5-Hydroxy-thiabendazole	7.9	218.0	191.0	147.0	31	37
Albendazole-sulfoxide	8.5	282.0	239.9	208.0	21	19
Amino-mebendazole	8.5	238.0	104.9	133.0	31	37
Amino-flubendazole	8.7	256.0	123.0	133.0	31	37
Albendazole-sulfone	8.8	298.0	265.9	159.1	26	29
Fenbendazole-sulfoxide	8.9	316.0	159.0	283.8	26	47
Thiabendazole	8.9	202.0	174.9	130.9	36	35
Fenbendazole-sulfone	9.1	332.0	299.9	159.1	31	31
Hydroxy-flubendazole	9.1	316.0	283.0	97.0	31	31
Cambendazole	9.2	303.0	216.9	260.8	36	37
Mebendazole	9.5	296.0	264.0	105.0	31	31
Flubendazole	9.6	314.0	281.9	123.0	31	31
Oxibendazole	9.6	250.0	218.1	176.1	26	25
Albendazole	10.0	266.0	234.0	191.0	26	29
Fenbendazole	10.2	300.0	267.8	159.0	31	29
Triclabendazole	11.0	359.0	273.9	171.0	31	53
Levamisole	7.7	205.0	178.1	123.0	26	29
Dichlorvos	8.2	220.9	109.0	126.9	31	25
Morantel	8.3	221.0	176.8	111.0	66	15
Haloxon	9.8	414.8	352.8	210.9	36	29
Coumaphos-oxon	9.9	346.9	290.9	318.9	26	25
Coumaphos	10.6	362.9	226.8	306.8	31	35
TPP (int std)	10.6	327.0	77.2	152.0	61	65
Cyprodinil (QC std)	10.9	226.0	108.0	106.0	30	35
ESI$^-$						
Clorsulon	8.7	377.7	341.6	241.6	−41	−16
Nitroxynil	8.9	288.9	126.7	161.8	−41	−34
2,4-D (int std)	9.1	218.8	160.8	124.9	−21	−18
Oxyclozanide	10.5	397.8	175.7	361.7	−41	−38
Triclabendazole-sulfoxide	10.6	372.7	180.8	212.7	−36	−60
Niclosamide	11.1	324.9	170.8	288.8	−36	−38
Closantel	11.2	660.9	126.7	344.7	−61	−78
Bithionol	11.4	352.8	160.8	191.7	−36	−32
Rafoxanide	11.9	623.9	126.8	344.6	−76	−70

[a] Most abundant fragment ion.

and an aliquot (10 μL) was injected onto the LC–MS/MS system.

2.5. Liquid chromatography

Reversed-phase separation of analytes was performed at 30 °C on a Prodigy™ C_{18} column (150 mm × 3 mm I.D., 5 μm particles) protected by a C_{18} guard column (4 mm × 3 mm I.D.), both from Phenomenex (Torrence, CA, USA). The final mobile phase conditions were (A) 25 mM aqueous ammonium formate at pH 4 and (B) 12.5 mM ammonium formate in MeCN:MeOH (50:50, v/v). Gradient elution (0.3 mL min^{-1}) was used with an initial starting condition of 98:2 (A:B, v/v), and the organic concentration increased to 100% over 5 min, where it was held for 10.5 min before it returned to the initial starting condition.

2.6. Mass spectrometry

MS analyses were performed by atmospheric pressure electrospray ionisation in the positive (ESI$^+$) and negative (ESI$^-$) modes applied in sequential injections using the same LC conditions. The eluent flow was diverted to waste for 5 min after sample injection followed by a second divert (after 15 min) to waste prior to the next chromatographic sequence to minimise column contamination. Nitrogen was used for nebulisation, desolvation and collision gases. The source temperature and ionspray voltage were set at 500 °C and ±4500 V, respectively. The analytes were detected by tandem MS using the multiple reaction monitoring (MRM) function of two transitions with a dwell time of 100 ms. In the ESI$^+$ method, the MS acquisition was divided into three time periods (7.0–11.8 min, 11.8–14.0 min, and 14.0–15.5 min). The ESI$^-$ method acquired the monitored ions in two time periods (8.0–9.5 min and 9.5–15.0 min). The MS conditions were optimised by tuning the analyte-specific parameters, including declustering potential, focusing potential, collision energy, and collision cell exit potential for each analyte. This optimisation was carried out by infusion of a 1 μg mL^{-1} standard solution of each analyte and monitoring the two most abundant fragment ions produced from the molecular ion. A summary of the retention times, monitored ions and optimised MS parameters obtained for each analyte is reported in Table 2.

2.7. Validation procedure

The LC–MS/MS method was validated according to 2002/657/EC guidelines. LC–MS/MS identification criteria were verified through-

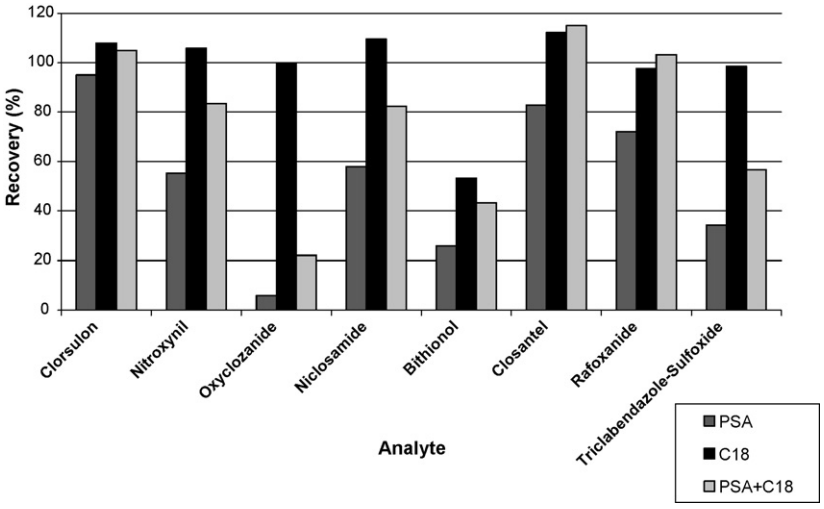


Fig. 2. Comparison of different sorbents for dispersive-SPE cleanup of eight analytes in milk (ESI−).

out the validation study by monitoring relative retention times, ion recognition (signal-to-noise ratio (S/N)) and relative ion intensities.

Several method validation parameters were determined including linearity, specificity, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (limit of quantitation, lowest calibrated level, decision limit CCα, and detection capability CCβ). The specificity of the method was verified through the analysis of 20 negative milk and liver samples obtained from different sources. Interferences from other analytes on identification and quantitation were checked by analysing negative samples fortified separately with each analyte at 100 μg kg^{-1}. To test the linearity of the method, negative samples were fortified at three concentration levels (5, 50, and 500 μg kg^{-1}) to give a four-point calibration curve (including 0 μg kg^{-1}) that covered the wide range of MRLs for the analytes.

Since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. The recovery and precision were determined through the analysis of negative milk and liver samples fortified in six replicates at 0.5, 1, 1.5 and 2 times the MRL (as listed in Table 1) or 10 μg kg^{-1} for analytes with no specified limit. To evaluate precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability results were obtained at the four validation levels under identical conditions by the same operator on three separate days (n = 18 each level overall). The within-laboratory reproducibility results were obtained at the four validation levels by different operators on three separate days (n = 18 each level overall). The decision limit (CCα) was calculated by analysing 20 negative milk and liver samples fortified at MRL (or 10 μg kg^{-1} levels), and taking the concentration at the permitted limit plus 1.64 times the standard deviation obtained. The detection capability (CCβ) of the proposed method was calculated from the CCα value plus 1.64 times the corresponding standard deviation. The LOQ was estimated for each analyte by injecting low concentration matrix-matched standards and measuring the S/N of the peaks. The lowest concentration that yielded S/N ≥ 10 was defined as the LOQ. The LOQ was also used as the lowest calibrated level (LCL) in the validation experiments.

Throughout the validation procedure, an internal standard solution (2,4-D and TPP) was added to each sample (100 μg kg^{-1}) prior to extraction and a QC standard (cyprodinil) was added prior to injection (100 μg kg^{-1}).

3. Results and discussion

3.1. Method development

3.1.1. Liquid chromatography–mass spectrometry

LC–MS/MS methods have been previously reported in literature to determine BZs and MLs using various mobile phase additives. BZs were reported to be ionised using formic acid [32] and a combination of ammonium acetate and acetic acid [33], while MLs were ionised with ammonium acetate [34] or acetic acid [35]. The LC–MS/MS method described in this paper is the first to achieve quantitative-confirmatory analysis of BZs, MLs, the largely ignored flukicides, and selected pesticides with anthelmintic activity. As a result, the mobile phase composition was carefully optimised to achieve efficient ionisation and separation of all 38 analytes.

Formic acid (0.1%) was initially evaluated as a mobile-phase additive but was found to be unsuitable due to the formation of sodium adducts for some of the analytes, namely abamectin, doramectin and ivermectin. Ammonium acetate and ammonium formate were evaluated as alternative additives and formed reproducible ions ($[M+H]^+$, $[M+NH_4]^+$ and $[M-H]^-$) for the 38 analytes. The ammonium salts also gave increased signal intensities (relative to formic acid) for most negatively ionised analytes but did not change positive ion intensities. Ammonium formate was included in the final mobile phase conditions because of its higher solubility compared to ammonium acetate in MeCN. The optimal mobile phase conditions were (A) 25 mM aqueous ammonium formate at pH 4 and (B) 12.5 mM ammonium formate in MeCN:MeOH (50:50, v/v). Mobile phase A was initially set at pH 7, but it was later found that a reduction to pH 4 increased the ion intensity for most positive ions and improved chromatography for levamisole and emamectin.

The instrument used in the study required 700 ms to switch between positive and negative modes and was found to be unsuitable to allow determination of all 38 analytes in a single injection. As a result, two injections were required to allow sensitive determination of the 30 ESI+ and 8 ESI− analytes for each sample. All 38 analytes eluted within 15.5 min, with a further 9 min period required for equilibration. A number of faster gradient conditions were investigated but resulted in analytes eluting too early, which led to matrix co-elution problems for sample extracts.

Table 3
Percentage of matrix co-extractives removed from bovine milk and liver.

Matrix	Concentration of co-extractives prior to clean up (mg g^{-1})	C_{18} (%)	PSA (%)	PSA + C_{18} (%)
Milk	16	73	10	90
Liver	31	36	81	89

According to 2002/657/EC, three identification points are required to satisfy confirmatory criteria for Group B substances. This was achieved through the selection of one precursor ion and two product ions, which resulted in four identification points, which exceeded the minimum requirements. 2,4-D and TPP were used as the internal standards in negative and positive modes, respectively. The fungicide, cyprodinil, was used as a QC standard to help isolate and assess LC–MS/MS performance.

3.1.2. Sample preparation

A QuEChERS-based approach was adapted to isolate the 38 analytes from milk and liver. The QuEChERS method has been successfully adapted and validated in multiple labs to extract hundreds of pesticides (including some of the BZs and organophosphates listed in Table 1) from various food matrices [20–28]. However, there are few reports of the application of this sample preparation approach in relation to veterinary drug residue analysis [24].

A simple QuEChERS extraction procedure was initially evaluated for isolating anthelmintic residues from fortified water or milk (10 ml) using MeCN (10 ml) as an extraction solvent with $MgSO_4$ (4 g) and NaCl (1 g) being employed as phase separation agents. In keeping with previous studies of pesticide analysis, MeCN was found to give satisfactory recovery (typically >90%) of the target analytes. The robustness of the extraction procedure was evaluated through varying the amounts of NaCl (0, 0.5, 1 and 2 g). Variation of the amounts of NaCl used for phase separation appeared to have little effect on analyte recovery. As a result, the original 1 g amount of NaCl was retained in the method because 50 mL centrifuge tubes containing 4 g $MgSO_4$ and 1 g NaCl were commercially available (solvent must be added before the sample if using them). Furthermore, NaCl helps to remove highly water-soluble polar matrix interferences, thus provides clean-up of the samples and reduces the need for frequent cleaning of the MS ionisation source.

The next step in sample preparation was to develop a dispersive SPE clean-up step through evaluation of the effects of different sorbent materials. In the original QuEChERS method, PSA was found to effectively remove fatty acids from food samples, while giving high recovery for a range of pesticides. In this study, the effects of three different sorbent materials, namely, PSA, GCB and C_{18} were investigated. PSA was found to give suitable recovery of positive mode analytes but lower recovery of negative mode analytes (see Fig. 2). C_{18} gave the highest recovery for the negative mode analytes and satisfactory recovery of positive mode analytes. A mixture of PSA and C_{18} was also investigated but gave lower recovery than C_{18}

Table 4
Recovery, repeatability, and within-laboratory reproducibility results for bovine milk at MRL level.

Analyte	Recovery		Repeatability		Within-lab reproducibility	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Bithionol	94	4	108	17	104	16
Clorsulon	109	4	99	13	101	12
Closantel	89	4	85	12	86	11
Nitroxynil	99	4	98	12	94	11
Niclosamide	106	4	92	12	96	12
Rafoxanide	66	6	66	12	66	11
Oxyclozanide	115	4	196	36	176	40
Triclabendazole	78	9	87	12	88	11
Triclabendazole-sulphoxide	87	5	87	12	87	10
Albendazole	104	2	102	7	104	7
Albendazole-sulphoxide	88	3	93	6	95	6
Albendazole-sulphone	98	2	105	6	105	6
Albendazole-2-amino-sulphone	85	3	90	6	91	6
Cambendazole	94	2	97	4	98	4
Fenbendazole	70	5	75	8	77	8
Fenbendazole-sulphoxide	61	4	88	24	91	21
Fenbendazole-sulphone	98	3	101	7	100	6
Flubendazole	93	2	98	8	100	8
Amino-flubendazole	79	5	88	10	89	9
Hydroxyl-flubendazole	91	4	104	11	107	12
Mebendazole	103	3	101	6	102	6
Amino-mebendazole	80	7	87	9	88	8
Oxibendazole	61	5	86	25	90	22
Thiabendazole	78	3	85	8	87	9
5-Hydroxy-thiabendazole	101	5	106	7	106	7
Abamectin	83	3	88	6	90	6
Doramectin	86	2	91	5	91	5
Emamectin	78	3	82	6	83	6
Eprinomectin	86	2	88	4	89	4
Ivermectin	80	3	84	5	86	5
Moxidectin	79	3	87	7	88	7
Selamectin	86	2	89	4	90	4
Coumaphos	86	12	99	13	99	12
Coumaphos-oxon	95	5	83	30	91	28
Dichlorvos	68	27	198	98	176	96
Haloxon	79	18	91	18	99	21
Levamisole	82	3	86	6	89	7
Morantel	82	3	88	7	90	8

alone. GCB was also considered as a sorbent, by itself and with a mixture of PSA and C_{18}. GCB appeared to give excellent cleanup of matrix co-extractives but was found to be unsuitable in this application because it also retained the structurally planar analytes, resulting in low recovery for most analytes.

An experiment was conducted to determine what percentage of matrix co-extractives was removed from the samples and therefore which sorbent provided the most effective clean-up. This involved extracting 10 g milk or liver (in triplicate for each sorbent) with MeCN and performing dispersive-SPE with 7.5 mL of the extract. 5 mL of the purified extract was transferred to a pre-weighed glass tube and evaporated to dryness. The sample tubes were re-weighed and the amount of co-extractives calculated by difference. The effectiveness of the sorbents was determined by comparing the results for the samples that underwent clean-up with those that did not undergo clean-up. Table 3 lists the results in a comparison of C_{18}, PSA and a mixture of PSA + C_{18}. As shown previously for milk and other fat-containing matrices in pesticide analysis [25], a combination of PSA + C_{18} gave the most efficient clean-up of the samples with approximately 90% of matrix co-extractives removed. PSA gave sufficient clean-up of liver while C_{18} gave better clean-up for milk.

Due to the low recovery obtained for the negative-mode analytes with PSA, C_{18} was found to be the most suitable sorbent for the method, given its ability to provide satisfactory clean-up while achieving high recoveries for all 38 analytes.

3.2. Validation

3.2.1. Liquid chromatography–mass spectrometry

The criteria on relative retention times (RRTs) and ion ratios (IRs) were examined for all samples and standards used for the validation study. The values for RRT and IR were in agreement with the EU requirements [31] for all the analyses investigated in the study. In terms of RRT, the analyte peaks in samples were found to be within the ±2.5% tolerance when compared with standards. Furthermore, two transition ions were monitored for each of the 38 analytes, although only the most intense ion was used as the quantitation ion. All ion ratios of samples were within the required tolerances as required by EU criteria when compared with standards during the validation study.

3.2.2. Linearity

The linearity was demonstrated for all 38 analytes by preparing a four-point matrix-matched calibration curve at concentration levels of 0, 5, 50 and 500 $\mu g\,kg^{-1}$. The majority of analytes gave linear regression values greater than 0.99. The only exception was nitroxynil in milk, which had a linear regression value greater than 0.98.

3.2.3. Selectivity

The selectivity of the method was demonstrated through the analysis of milk ($n = 20$), and liver ($n = 20$) samples from

Table 5
Recovery, repeatability and within-laboratory reproducibility results for bovine liver at MRL level.

Analyte	Recovery		Repeatability		Within-lab reproducibility	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Bithionol	51	21	66	26	66	26
Clorsulon	95	5	106	14	106	13
Closantel	96	3	109	21	106	19
Nitroxynil	55	21	65	16	68	18
Niclosamide	87	3	88	15	85	12
Rafoxanide	86	4	84	17	82	15
Oxyclozanide	106	3	95	16	92	19
Triclabendazole	93	4	94	9	94	9
Triclabendazole-sulphoxide	111	3	92	19	100	20
Albendazole	96	4	95	4	115	32
Albendazole-sulphoxide	83	5	83	7	77	15
Albendazole-sulphone	95	2	97	6	94	8
Albendazole-2-amino-sulphone	66	3	62	8	87	51
Cambendazole	99	3	93	10	101	15
Fenbendazole	77	2	84	8	87	10
Fenbendazole-sulphoxide	88	2	89	6	85	11
Fenbendazole-sulphone	95	2	100	7	97	9
Flubendazole	97	5	99	8	99	7
Amino-flubendazole	68	8	60	17	83	51
Hydroxyl-flubendazole	102	9	93	14	96	14
Mebendazole	90	2	89	6	89	6
Amino-mebendazole	70	10	59	17	80	49
Oxibendazole	97	5	93	8	100	15
Thiabendazole	90	3	85	7	90	12
5-Hydroxy-thiabendazole	87	2	70	20	69	17
Abamectin (B_{1a})	94	5	93	9	91	9
Doramectin	72	8	74	9	71	11
Emamectin (B_{1a})	95	3	83	20	80	19
Eprinomectin (B_{1a})	97	3	98	9	95	11
Ivermectin (H_2B_{1a})	73	4	70	11	67	12
Moxidectin	92	7	97	10	92	14
Selamectin	82	4	76	14	79	13
Coumaphos	128	10	117	23	117	21
Coumaphos-oxon	91	6	80	12	81	13
Dichlorvos	106	12	93	18	92	18
Haloxon	96	15	75	25	82	26
Morantel	93	2	90	6	91	6
Levamisole	78	5	76	5	78	6

Table 6
Lowest calibrated level (LCL), decision limit (CCα), detection capability (CCβ) and MRL for the analytes in bovine milk and liver.

Analyte	LCL (μg kg^{-1})		CCα (μg kg^{-1})		CCβ (μg kg^{-1})		MRL[a] (μg kg^{-1})	
	Milk	Liver	Milk	Liver	Milk	Liver	Milk	Liver
Bithionol	5	5	12	15	15	19	10	10
Clorsulon	5	5	11	117	12	135	10	100
Closantel	5	5	12	1228	13	1455	10	1000
Nitroxynil	5	5	11	24	11	29	10	20
Niclosamide	5	5	11	11	11	13	10	10
Rafoxanide	5	5	14	11	17	13	10	10
Oxyclozanide	5	5	20	623	31	746	10	500
Triclabendazole	5	5	11	282	13	315	10	250
Triclabendazole-sulphoxide	5	5	11	314	11	378	10	250
Albendazole	5	5	108	1383	117	1766	100	1000
Albendazole-sulphone	5	5	108	1099	116	1198	100	1000
Albendazole-sulphoxide	5	5	108	1149	117	1290	100	1000
Albendazole-2-amino-sulphone	5	5	108	1077	117	1154	100	1000
Cambendazole	5	5	11	12	11	14	10	10
Fenbendazole	5	5	11	568	12	636	10	500
Fenbendazole-sulphoxide	5	5	12	555	14	611	10	500
Fenbendazole-sulphone	5	5	11	559	11	619	10	500
Flubendazole	5	5	11	11	12	11	10	10
Amino-flubendazole	5	5	11	14	12	18	10	10
Hydroxy-flubendazole	5	5	12	12	13	14	10	10
Mebendazole	5	5	11	11	12	12	10	10
Amino-mebendazole	5	5	11	14	12	18	10	10
Oxibendazole	5	5	13	12	15	13	10	10
Thiabendazole	5	5	111	116	123	133	100	100
5-Hydroxy-thiabendazole	5	5	112	118	124	136	100	100
Abamectin	5	5	11	22	11	25	10	20
Doramectin	5	5	11	111	11	122	10	100
Emamectin	5	5	11	12	11	14	10	10
Eprinomectin	5	5	21	1721	22	1943	20	1500
Ivermectin	5	5	11	112	11	124	10	100
Moxidectin	5	5	43	116	45	132	40	100
Selamectin	5	5	11	11	11	12	10	10
Coumaphos	5	5	11	13	13	17	10	10
Coumaphos-oxon	5	5	13	11	16	13	10	10
Dichlorvos	10	10	38	12	65	15	10	10
Haloxon	5	5	12	13	15	16	10	10
Levamisole	5	5	11	107	12	113	10	100
Morantel	5	5	55	847	61	893	50	800

[a] See Table 1 for MRLs (10 μg kg^{-1} for analytes without an MRL).

different sources, which were found to be free of interference from endogenous materials. The specificity of the assay was demonstrated through separate injection of individual standards and checking interfering peaks at the retention time of other analytes. This experiment showed no interfering peaks co-eluting with analytes. Due to the high selectivity of the MS in MRM mode, peaks that eluted close together could be clearly distinguished based on their different precursor and product ions.

3.2.4. Recovery

The results of the recovery experiments are given in Tables 4 and 5. The majority of analytes met EU validation requirements with recovery values typically in the range 70–120% in milk and liver, and relatively few did not meet this range, as discussed below. The vast majority of analytes also gave <10% R.S.D. ($n = 6$) at each spiking level. Only a small number of analytes gave >15% R.S.D. Small differences between the two matrices can be observed in Tables 4 and 5, with milk, in general, giving better results than liver. This is probably due to liver being a more complicated matrix with more co-extractives (see Table 3).

In milk, rafoxanide gave 66% recovery (6% R.S.D.), indicating that some loss occurs during sample preparation, although the somewhat lower recovery with satisfactory repeatability is not particularly problematic. The method LOQ for rafoxanide remained <5 μg kg^{-1}. On the other hand, dichlorvos gave average recovery of 68% with R.S.D. of 27%. This more variable result was due to the lower sensitivity of the MS/MS method for dichlorvos than the other analytes, and the fortification of dichlorvos close to its LOQ in the validation study. Dichlorvos had an LCL of 10 μg kg^{-1} in matrix-matched calibration standards whereas all the other analytes could be quantified at less than 5 μg kg^{-1}. As a result, dichlorvos gave worse results in terms of recovery, repeatability, within-laboratory reproducibility, CCα, and CCβ in comparison to the other 37 analytes.

In liver, recovery for five analytes was slightly outside the acceptable limits. Recovery for negative-mode analytes, bithionol and nitroxynil, was 51% and 55%, respectively, with R.S.D. of 21% for both, this compares to a recovery of 100% observed in milk. Since matrix-matching calibration was used (and ESI− showed no ion suppression or enhancement effects), the poorer results in liver were either due to the partial degradation or greater interaction of the analytes with the more complex liver matrix. The ESI+ mode analytes, albendazole-2-amino-sulphone and amino-flubendazole gave reasonably acceptable results (like rafoxanide in milk) with 66% and 68% recovery and 3% and 8% R.S.D.s, respectively. Lastly, coumaphos gave an elevated recovery of 128% with 10% R.S.D., which indicates a high bias from an unknown source in this isolated case.

3.2.5. Precision

Two parameters were investigated for precision, namely repeatability and within-laboratory reproducibility, reported in terms of relative standard deviations which appear in Tables 4 and 5. In milk, the majority of analytes gave <15% R.S.D. ($n = 18$), while in liver the vast majority gave <20% R.S.D. ($n = 18$). A small number of analytes did not meet the desirable R.S.D. level (<23%), which is further discussed below. Good recoveries (70–120%) were obtained by analysts in both matrices. Liver gave an average overall recovery of 88%, while the average overall recovery for milk was 97%. Again, a difference was observed in the results for the two matrices, with milk giving better results than liver.

In milk, there were five analytes with results outside the acceptable EU regulatory limits for precision. The two most problematic analytes were dichlorvos and oxyclozanide. The problem with dichlorvos has already been discussed, and in the case of oxyclozanide, excessively high recovery (196% and 176%) and R.S.D.s (36% and 40%) were obtained for the repeatability and within-lab reproducibility, respectively. The elevated results were due to two experiments (out of six) showing very high area counts for oxyclozanide and dichlorvos, but not the remaining 36 analytes. This demonstrated that the problem was compound specific, and the increased area counts for oxyclozanide were probably due to an isolated ion enhancement effect (in ESI−, MRL level). Fenbendazole-sulphoxide (24% R.S.D.) and oxibendazole (25% R.S.D.) gave slightly elevated results for the repeatability, while coumaphos-oxon gave 30% R.S.D. for repeatability and 28% R.S.D. for within-lab reproducibility. These results do not meet the criteria established by EU single-laboratory validation criteria for a quantitative confirmation method, but periodic outliers like this are expected in multi-class, multi-residue analysis.

In liver, there were six analytes that displayed results outside the acceptable limits for EU regulatory monitoring. The problematic analytes in the within-lab reproducibility experiment were albendazole (32% R.S.D.), albendazole-2-amino-sulphone (51%), amino-flubendazole (51% R.S.D.) and amino-mebendazole (49% R.S.D.). However, they gave good results in both repeatability experiments. Furthermore, bithionol and haloxon gave slightly elevated R.S.D.s for repeatability and reproducibility (25–26%) in all cases. Dichlorvos unexpectedly gave good results for this part of the validation in liver, with 93% recovery and 18% R.S.D.

3.2.6. Analytical limits

The analyte LOQs were estimated by analysing matrix-matched standards at low concentrations and checking for peak signals with an S/N greater than 10. The 5 $\mu g\,kg^{-1}$ fortified samples were found to have S/N greater than 10 for all analytes, except dichlorvos (LOQ $\approx 10\,\mu g\,kg^{-1}$). The higher LOQ for dichlorvos than the other analytes by LC–MS/MS is in keeping with previous experience [28]. Otherwise, all of the other analytes gave LOQ < 5 $\mu g\,kg^{-1}$. There is no regulatory need to quantify the analytes at lower concentrations, and 5 $\mu g\,kg^{-1}$ was set as the LCL (see Table 6). This LCL is below the MRL for all 38 anthelmintics in milk or liver, with the lowest MRL being 10 $\mu g\,kg^{-1}$ (see Table 1), including analytes which are not allowed in lactating species.

The CCα and CCβ values were calculated at MRL level for analytes with an established permitted limit and at 10 $\mu g\,kg^{-1}$ for those analytes without an established limit. Both sets of concentration values were determined by analyzing 20 negative samples fortified at the levels stated in Table 1 prior to extraction. The values were calculated using the results already obtained for the repeatability and within-laboratory reproducibility studies. The concentration at the MRL (or 10 $\mu g\,kg^{-1}$) plus 1.64 times the calculated standard deviation equals CCα and CCβ includes the addition of another factor of 1.64 times the standard deviation. Table 6 presents the CCα and CCβ results for the analytes in the study for milk and liver.

4. Conclusions

An efficient and effective QuEChERS-based LC–MS/MS multiclass, multi-residue method has been developed for the determination of 38 major anthelmintic veterinary drugs in bovine milk and liver. The method achieves high quality of results (good recovery, repeatabilities, within-lab reproducibilities, and wide analytical scope) and practical benefits (low cost, high sample throughput, little labour, hardly any waste and few labware and space demands). The method has been single laboratory validated according to the 2002/657/EC guidelines and met acceptability criteria in all but a few cases. The method was found to be very sensitive and gave LOQ < 5 $\mu g\,kg^{-1}$ for all but one analyte which had LOQ $\approx 10\,\mu g\,kg^{-1}$.

Acknowledgements

This research was funded by the Irish Department of Agriculture and Food under the Food Institutional Research Measure as part of the National Development Plan, and by the United States Agency of International Development, grant TA-MOU-03-CA23-005.

Additionally, we acknowledge Merial, Janssen Animal Health and Pfizer Animal Health for donating standards.

References

[1] R.J. Horton, Parasitol. Today 6 (1990) 106.
[2] A.F. Loyacano, J.C. Williams, J. Guriea, A.A. De Rosa, Vet. Parasitol. 107 (2002) 227.
[3] J. Vercruysse, P. Dorny, Int. J. Parasitol. 29 (1999) 165.
[4] Q.A. McKellar, E.W. Scott, J. Vet. Pharmacol. Ther. 13 (1990) 223.
[5] F.K. Mohammed, G.A.-M. Faris, M.S.H. Rhayma, K. Ahmed, Neurotoxicology 27 (2006) 153.
[6] G.R. Lankas, C.P. Peter, Food Chem. Toxicol. 30 (1992) 297.
[7] European Agency for the Evaluation of Medicinal Products, EMEA/MRL/452/98-FINAL (1998) Nitroxynil summary report.
[8] S.G. Vega, P. Guzman, L. Garcia, J. Espinosa, C. Cortinas de Nava, Mutat. Res. 204 (1988) 269.
[9] M. Danaher, H. De Ruyck, S.R.H. Crooks, G. Dowling, M. O'Keeffe, J. Chromatogr. B 845 (2007) 1.
[10] M. Danaher, L.C. Howels, S.R.H. Crooks, V. Cerkvenik-Flajs, M. O'Keeffe, J. Chromatogr. B 844 (2006) 175.
[11] K. Takeba, T. Itoh, M. Matsumoto, J. AOAC Int. 79 (1996) 848.
[12] E.C. von Tonder, M.M. de Villiers, J.S. Handford, C.E.P. Malan, J.L. du Preez, J. Chromatogr. A 729 (1996) 267.
[13] C.P. Talley, N.R. Trenner, G.V. Downing Jr., W.J.A. Vanden Heuvel, Anal. Chem. 43 (1971) 1379.
[14] W.J. Blanchflower, D.G. Kennedy, Analyst 114 (1989) 1013.
[15] G. Stoev, T. Dakova, A. Michailova, J. Chromatogr. A 846 (1999) 383.
[16] T.A. Wehner, J.S. Wood Jr., R. Walker, G.V. Downing, W.J.A. Vanden Heuvel, J. Chromatrogr. A 399 (1987) 251.
[17] A.R. Khan, M.J. Akhtar, R. Mahmood, S.M. Ahmed, S. Malook, M. Iqbal, J. Pharm. Biomed. Anal. 22 (2002) 111.
[18] K. Takeba, K. Fujinuma, M. Sakamoto, T. Miyazaki, H. Oka, Y. Itoh, H. Nakazawa, J. Chromatogr. A 882 (2000) 99.
[19] C. Palma, C. Godoy, M. Arboix, Arch. Med. Vet. 38 (2006) 265.
[20] M. Anastassiades, S.J. Lehotay, D. Štajnbaher, F.J. Schenk, J. AOAC Int. 86 (2003) 412.
[21] S.J. Lehotay, A. de Kok, M. Hiemstra, P. van Bodegraven, J. AOAC Int. 88 (2005) 595.
[22] P. Payá, M. Anastassiades, D. Mack, I. Sigalova, B. Tasdelen, J. Oliva, A. Barba, Anal. Bioanal. Chem. 389 (2007) 1697.
[23] F. Plössl, M. Giera, F. Bracher, J. Chromatogr. A 1135 (2006) 19.
[24] A. Posyniak, J. Zmudzki, K. Mitrowska, J. Chromatogr. A 1087 (2005) 259.
[25] S.J. Lehotay, K. Mastovska, S.-J. Yun, J. AOAC Int. 88 (2005) 630.
[26] T.D. Nguyen, B.S. Lee, B.R. Lee, D.M. Lee, G.H. Lee, Rapid Commun. Mass Spectrom. 21 (2007) 3115.
[27] K. Mastovska, S.J. Lehotay, Agric. Food Chem. 54 (2006) 7001.
[28] S.J. Lehotay, J. AOAC Int. 90 (2007) 485.
[29] A.A. Stolker, U.A. Brinkman, J. Chromatogr. A 1067 (2005) 15.
[30] M. Hiemstra, A. de Kok, J. Chromatogr. A 1154 (2007) 3.

[31] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results, Off. J. Eur. Commun. L221 (2002) 8.
[32] H. De Ruyck, E. Daeseleire, H. De Ridder, R. Van Renterghem, J. Chromatogr. A 976 (2002) 181.
[33] G. Balizs, J. Chromatogr. B 727 (1999) 167.
[34] R. Sheridan, J. AOAC Int. 89 (2006) 1088.
[35] M.D. Hernando, J.M. Suarez-Barcena, M.J.M. Bueno, J.F. Garcia-Reyes, A.R. Fernandez-Alba, J. Chromatogr. A 1 (2007) 62.